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NEW APPLICATION TRANSMITTAL

Transmitted herewith for filing is the patent application of Peter X. Ma for **THREE-DIMENSIONAL HYDROGEL/CELL SYSTEM**.

CERTIFICATION UNDER 37 C.F.R. § 1.10

I hereby certify that this New Application Transmittal and the documents referred to as enclosed therein are being deposited with the U.S. Postal Service on this date **February 23, 1999** in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number **EL 079 494 398 US** addressed to: **Box Patent Application**, Assistant Commissioner For Patents, Washington, D.C. 20231.

Marlene Garitano

Marlene Garitano

1. **Type Of Application**
This new application is for a(n)
☒ Original (nonprovisional)
2. **Papers Enclosed That Are Required For Filing Date Under 37 C.F.R. § 1.53(b) (Regular) or 37 C.F.R. § 1.153 (Design) Application**
17 Pages of Specification
3 Pages of Claims
1 Page of Abstract
11 Sheets of Informal Drawings
3. **Declaration**
☒ Enclosed
☒ Unexecuted.
4. **Inventorship Statement**
The inventorship for all the claims in this application is:
☒ the same
5. **Language**
☒ English
6. **Assignment**
☒ An assignment of the invention to **The Regents Of The University of Michigan** is attached.
☒ Form PTO-1595 will follow.
7. **Fee Calculation (37 C.F.R. § 1.16)**
☒ Regular application

CLAIMS AS FILED

Number Filed	Number Extra	Rate	Basic Fee - \$760.00 (37 C.F.R. § 1.16(a))
Total Claims (37 C.F.R. § 1.16(c))	20 - 20 =	0 × \$18.00 =	\$0.00
Independent Claims (37 C.F.R. § 1.16(b))	3 - 3 =	0 × \$78.00 =	\$0.00
Multiple Dependent Claim(s), if any (37 C.F.R. § 1.16(d))	+ \$260.00 =		\$0.00
Filing Fee Calculation			\$760.00

8. **Small Entity Statement(s)**

- ☒ Verified Statement(s) that this is a filing by a small entity under 37 C.F.R. §§ 1.9 and 1.27 is(are) attached.

Filing Fee Calculation (50% of above) \$380.00

9. **Fee Payment Being Made At This Time**

- ☒ Enclosed

☒ basic filing fee

\$380.00

Total Fees Enclosed \$380.00

10. **Method of Payment of Fees**

- ☒ Check in the amount of \$380.00

11. **Authorization To Charge Additional Fees and Credit Overpayment**

- ☒ The Commissioner is hereby authorized to charge payment of any fees associated with this communication or credit any overpayment to Deposit Account No.: **08-1290**. An originally executed duplicate of this transmittal is enclosed for this purpose.

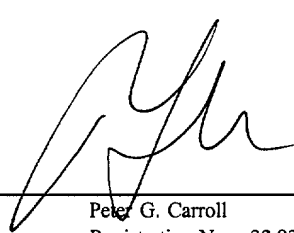
12. **Power of Attorney by Assignee**

- ☒ Enclosed

13. **Return Receipt Postcard**

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☒ **Statement Where No Further Pages Added**

- ☒ This transmittal ends with this page.

Applicant / Patentee: Peter X. Ma
For: **THREE-DIMENSIONAL HYDROGEL/CELL SYSTEM**

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR §§ 1.9(f) and 1.27(d) - NONPROFIT ORGANIZATION)**

I hereby declare that I am an official empowered to act on behalf of the university (or other institution of higher education) identified below:

The Regents Of The University of Michigan
3003 South State Street
Ann Arbor, Michigan 48109-1280

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR § 1.9(e) for purposes of paying reduced fees under §§ 41(a) and (b) of Title 35, United States Code with regard to the invention entitled **THREE-DIMENSIONAL HYDROGEL/CELL SYSTEM**, by inventor named **Peter X. Ma**, described in the specification filed herewith.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR § 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Date: _____

By: _____

Name: _____

Title: _____

THREE-DIMENSIONAL HYDROGEL/CELL SYSTEM

The present application claims priority to U.S. Provisional Patent Application Serial No. 60/075,802, filed February 24, 1998.

FIELD OF THE INVENTION

5 The present invention relates to compositions and methods for growing cells in a three-dimensional hydrogel/cell system, and in particular, for growing cells for the fabrication of tissues and organs.

BACKGROUND

10 Every year, millions of Americans suffer tissue loss or end-stage organ failure. Approximately 8 million surgical procedures are performed annually in the United States to treat these disorders. Physicians treat organ or tissue loss by transplanting organs from one individual to another. Although transplantation is one of the life-saving therapies, it is seriously limited by donor scarcity. Tissue engineering, which aims at creating biological body parts as alternatives for transplants, offers the possibility of substantial savings by providing substitutes that are less expensive than
15 donor organs and by providing a means of intervention before patients become critically ill (Langer and Vacanti, "Tissue engineering," Science 260: 920-926 [1993]).

20 One approach for tissue engineering uses tissue-inducing substances. The success of this approach depends on the purification and large-scale production of appropriate signal molecules, such as growth factors, and, in many cases, the development of methods to deliver these molecules to their targets. Another approach uses isolated cells or cell substitutes. This approach avoids the complications of surgery, allows replacement of only those cells that supply the needed function, and permits manipulation of cells before infusion.

25 However, isolated cells cannot form new tissues on their own. Most cells have a requirement for attachment to a surface in order to replicate and function, and require specific environments which often include the presence of supporting material

to act as a template for growth. Three dimensional scaffolds are used to mimic their natural counterparts, the extracellular matrices of the body. They serve both as a physical support and as an adhesive substrate (U.S. Patent 5,514,378 to Mikos *et al.* [1996]). Thus, scaffolding plays a pivotal role in the engineering of new tissues and organs (Ma and Langer, "Methods for the fabrication of biodegradable polymer foams for cell transplantation and tissue engineering," in Tissue Engineering Methods and Protocols, Yarmush and Morgan (eds.), Humana Press Inc.: Totowa, NJ, 1998).

Natural or synthetic polymers can be used to form highly porous scaffolds. Various tissues have been engineered from highly porous scaffolds prepared from synthetic biodegradable polymers such as poly(glycolic acid), poly(lactic acid), and poly(glycolic acid-co-lactic acid). (*See e.g.*, Ma and Langer, *supra*; Ma *et al.*, J. Biomed Mater. Res. 29: 1587-1595 [1995]; Ma *et al.*, Ann. Thorac. Surg. 60: S513-516 [1995]; Cusick *et al.*, J. Pediatr. Surg. 32: 357-360 [1997]; Ma *et al.*, Transactions of the Society for Biomaterials 295 [1997]; Ma and Langer, "Degradation, structure and properties of fibrous nonwoven poly(glycolic acid) scaffolds for tissue engineering," in Polymers in Medicine and Pharmacy, Mikos *et al.* (eds.), pp. 99-104, MRS: Pittsburg [1995]; Shinoka *et al.*, Circulation 94 (9 Supplement): II-164-168 [1996]; Cao *et al.*, Transplant Proc. 26: 3390-3392 [1994]; Kim *et al.*, Transplant Proc. 29: 858-860 [1997]). Although synthetic polymers generally give good reproducibility and controlled release kinetics compared to natural materials (*See e.g.*, U.S. Patent Nos. 5,855,610 and 5,716,404 to Vacanti *et al.*), they may not interact with cells in a desired manner (Langer and Vacanti, *supra*). On the other hand, natural polymers are advantageous in that they contain information (*e.g.*, particular amino acid sequences) that facilitates cell attachment or maintenance of differentiated function (Langer and Vacanti, *supra*).

What is needed is a method for controlling three-dimensional structures of hydrogel/cell constructs from natural polymers, for use as highly porous scaffolds that permit the support of cells for growth.

SUMMARY OF THE INVENTION

The present invention relates to compositions and methods for growing cells in a three-dimensional hydrogel/cell system, and in particular, for growing cells for the fabrication of tissues and organs. In one embodiment, the present invention
5 contemplates a method wherein ionically crosslinked alginate gels are used as scaffolds with defined dimensions for *in vitro* tissue engineering applications.

In a specific embodiment, the present invention contemplates a method for tissue engineering *in vitro* comprising the steps of: a) providing: i) an alginate salt, ii) a source of calcium ions, and iii) a calcium releasing compound; b) mixing the
10 alginate salt with the source of calcium ions to provide a mixture; and c) adding calcium releasing compound to provide a three-dimensional crosslinked hydrogel system. In one embodiment, the method further comprises the step of d) culturing the three-dimensional crosslinked hydrogel system for growing cells *in vitro*. While the above-named components can be added together, it is preferred that the method is
15 carried out in a stepwise fashion, such that said calcium releasing compound is added last. This has been found to produce the best results.

It is not intended that the present invention be limited by the nature of the alginate salt. Moreover, the present invention contemplates the combination of alginate materials and is not limited to one specific type of alginate salt. In one
20 embodiment, the alginate salt is sodium alginate. In another embodiment, the alginate salt is potassium alginate. In one embodiment, the alginate salt is prepared from an alginate source selected from *Macrocystis pyrifera* and *Laminaria hyperborea*. A variety of alginates (*e.g.*, from *Macrocystis pyrifera*) are commercially available (*e.g.*, from Sigma, St. Louis, MO).

It is not intended that the present invention be limited by the nature of the source of calcium ions. In one embodiment, the source of calcium ions is calcium carbonate. In another embodiment, the source of calcium ions is calcium sulfate. In
25 yet another embodiment, the source of calcium ions is calcium sulfate dihydrate. It is also not intended that the present invention be limited to a particular type of cation for

crosslinking (*i.e.*, calcium). In one embodiment, the present invention contemplates the use of divalent or multivalent cations as sources of cations for crosslinking. Moreover, the present invention contemplates the combination of cation sources for crosslinking.

5 It is not intended that the present invention be limited to the calcium releasing compound. The calcium releasing compound need simply cause the calcium source to release calcium ions, thereby initiating gelation. In one embodiment, the releasing compound comprises D-glucono- δ -lactone ($C_6H_{10}O_6$).

10 In one embodiment, the source of calcium ions is calcium carbonate, and the calcium-releasing compound is D-glucono- δ -lactone. Although it is not intended that the reagents be mixed using a particular ratio of reagents, the molar ratio of calcium carbonate to D-glucono- δ -lactone in one preferred embodiment is 0.5.

In yet another embodiment, the method of the present invention further comprises the step of implanting the three-dimensional crosslinked hydrogel system.

15 It is not intended that the three-dimensional crosslinked hydrogel system of the present invention be limited to a particular dimension. In one embodiment, the three-dimensional crosslinked hydrogel system has a thickness of between about 4 mm and about 8 mm. In another embodiment, the three-dimensional crosslinked hydrogel system has a diameter of approximately 18 mm.

20 It is also not intended that the three-dimensional crosslinked hydrogel system of the present invention have a particular composition. In one embodiment, the three-dimensional crosslinked hydrogel system has a calcium ion to carboxyl molar ratio of 0.27.

25 The present invention also contemplates the addition of other components (*e.g.*, inert or bioactive) without affecting the methods of the present invention.

30 In another embodiment, the present invention contemplates a method for tissue engineering *in vitro* comprising the steps of: a) providing: i) cells, ii) an alginate salt, iii) a source of calcium ions, and iv) a calcium releasing compound; b) mixing the cells, alginate salt, and the source of calcium ions to provide a mixture; c) adding the calcium releasing compound to the mixture to provide a crosslinked gel; and d)

culturing the crosslinked gel to provide a three-dimensional crosslinked hydrogel/cell system for growing cells *in vitro*.

In one embodiment, the alginate salt is selected from the group consisting of sodium alginate and potassium alginate. In another embodiment, the alginate salt is prepared from an alginate source selected from *Macrocystis pyrifera* and *Laminaria hyperborea*. In yet another embodiment, the source of calcium ions is selected from the group consisting of calcium carbonate and calcium sulfate. In an alternative embodiment, the calcium releasing compound is D-glucono- δ -lactone.

In one embodiment, the source of calcium ions is calcium carbonate, and the calcium releasing compound is D-glucono- δ -lactone, and the molar ratio of calcium carbonate to D-glucono- δ -lactone in one preferred embodiment is 0.5.

In another embodiment, the method further comprises the step of implanting said three-dimensional crosslinked hydrogel/cell system.

In one embodiment, the three-dimensional crosslinked hydrogel/cell system has a thickness of between about 4 mm and about 8 mm, and a diameter of approximately 18 mm. In another embodiment, the three-dimensional crosslinked hydrogel/cell system has a calcium ion to carboxyl molar ratio of 0.27.

It is not intended that the present invention be limited for culturing particular type of cells (or merely one cell type on a scaffold). A variety of cell types (including mixtures of different cells) are contemplated. In one embodiment, the cells are osteoblasts. In another embodiment, the cells secrete a medically useful compound (*e.g.*, hormone, cytokine, etc.). Such cells may be (but need not be) cells that have been manipulated by recombinant means to secrete such compounds.

Finally, the present invention also contemplates the resulting crosslinked alginate gel as a composition. Moreover, the present invention contemplates the resulting crosslinked gel in combination with other components, such as cells. It is not intended that the cells be limited to particular cell type, or merely one cell type on a scaffold. A variety of cell types, including mixtures of different cells, are contemplated.

DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

The present invention contemplates implanting crosslinked gels into hosts. The term "host" refers to both humans and animals.

"Initiating a reaction" means causing a reaction to take place. Reactions can be initiated by any means (*e.g.*, heat, wavelengths of light, addition of a catalyst, etc.) The present invention contemplates "initiating gelation" by addition of a calcium releasing compound to the above-described mixture, said mixture comprising a source of calcium ions.

As used herein, the term "implant" and "implanting" and the like indicates placement on, in, or through a patient's body (including placement in body cavities) in the course of medical treatment, *e.g.*, for a disease, impairment or injury. Implants include, but are not limited to, implants for wound care, drug delivery, and bone replacement.

As used herein, the term "hydrogel" refers to a three-dimensional network of cross-linked hydrophilic polymers in the form of a gel substantially composed of water, and preferably, but not limited to gels. Hydrogels may have a net positive or negative charge, or may be neutral. A typical net negative charged matrix is alginate. Hydrogels carrying a net positive charge may be typified by extracellular matrix components such as collagen and laminin. An example of a net neutral hydrogel is highly crosslinked polyethylene oxide or polyvinyl alcohol.

As used herein, the term "crosslinked" or "cross-linking" refers to an attachment of two chains of polymer molecules by bridges, composed of either an element, a group, or a compound, that join certain atoms of the chains by primary chemical bonds. For example, polysaccharide molecules can cross-link to form stable gel structures (*i.e.*, dextran). Cross-linking can also be effected artificially, such as by adding a chemical substance (*i.e.*, a cross-linking agent) and exposing the mixture to heat, or by subjecting the polymer to high-energy radiation.

As used herein, the term "alginate" refers to any of several derivatives of alginic acid (*e.g.*, calcium, sodium, or potassium salts or propylene glycol alginate). These compounds are hydrophilic colloids obtained from seaweed.

DESCRIPTION OF THE DRAWINGS

5 Figure 1 is a graph showing the dry to wet weight ratios of MP alginate gels with varying alginate concentrations and 1X CaCO₃.

 Figure 2 is a graph showing the volume ratios of a gel to the alginate suspension to form the gel (gelation time 48 hours) versus calcium content.

10 Figure 3 is a graph showing the compressive modulus of alginate gels versus alginate concentration.

 Figure 4 is a graph showing the compressive strength of alginate gels versus alginate concentrations.

 Figure 5 is a graph showing the compressive modulus of alginate gels versus calcium content.

15 Figure 6 is a graph showing the compressive strength of alginate gels versus calcium content.

 Figure 7 is a graph showing the swelling ratio of 3.18% *LH* alginate gels with 1.5X CaCO₃ in saline.

20 Figure 8a-8d show the compressive modulus strengths of alginate gels versus alginate concentration.

DESCRIPTION OF THE INVENTION

25 The present invention relates to compositions and methods for growing cells in a three-dimensional hydrogel/cell system, and in particular, for growing cells for the fabrication of tissues and organs. The present invention demonstrates that alginates from various sources can be ionically crosslinked to form gels. Their structural stability, mechanical properties can be controlled by alginate source and concentration, calcium ion source and concentration, and gel preparation methods.

The methods of the present invention permit the formation and preparation of structurally homogeneous and mechanically strong alginate gels with defined dimensions, which can be used to incorporate living cells. The three dimensional gel structure with incorporated cells can be maintained in an *in vitro* tissue culture environment by adjusting calcium ion concentration in the culture medium. These results have demonstrated how ionically crosslinked alginate gels with defined three dimensional structure can be reliably used as a tissue engineering scaffold.

The scaffolds can be maintained *in vitro* as well as implanted in a host. After fulfilling the purpose as a matrix or scaffold, the hydrogels of the present invention can be dissolved or degraded, leaving nothing behind except the regenerated new tissues or organs. Because of the abundance in material sources, low prices, and easy processing, the system makes large-scale tissue engineering/manufacture feasible.

Alginate acid, a polysaccharide from seaweeds, is a family of natural copolymers of β -D-mannuronic acid and α -L-guluronic acid (Martinsen *et al.*, Biotechnology and Bioengineering 33: 79-89 [1989]; Draget *et al.*, Carbohydrate Polymers 14: 159-178 [1991]. Because of their biocompatibility, abundance in source, and low prices, they have been widely used in food industry as thickeners and emulsifying agents. They have also been processed into gel beads encapsulating living cells as a means of immunoprotection. (See *e.g.*, Cottrell and Kovacs, "Alginates," in Handbook of water-soluble gums and resins," Davidson (ed.), McGraw-Hill: New York [1980]; Aebischer *et al.*, Transplantation 58: 1275-1277 [1994]; U.S. Patent 5,834,001 to Dionne *et al.*).

Alginate gels have not been reported as scaffolds to engineer tissues *in vitro*, probably due to the difficulties in controlling the mechanical properties, shape and size in a cell culture environment. The present invention describe methods and compositions related to the use of ionically crosslinked alginate gels as scaffolds with defined dimensions for *in vitro* tissue engineering applications, that are controlled and reproducible.

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

I. Materials

Sodium alginate prepared from *Laminaria hyperborea* (LH), Protanal LF200, was from Pronova Biopolymer (Drammen, Norway). High viscosity sodium alginate prepared from *Macrocystis pyrifera* (MP), calcium carbonate (CaCO_3), calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), calcium sulfate dihydrate ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$), D-glucono- δ -lactone ($\text{C}_6\text{H}_{10}\text{O}_6$) (GDL), L-ascorbic acid, dimethyl sulfoxide, and trypsin-EDTA were from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and phosphate-buffered saline (DPBS), fetal bovine serum (FBS), and penicillin-streptomycin (P/S) were from GibcoBRL (Grand Island, NY). MC3T3-E1 osteoblasts were kindly provided by Dr. R. Franceschi (University of Michigan School of Dentistry).

II. Methods

A. Gel Preparation

Sodium alginate was dissolved in either deionized water or a tissue culture medium. Either calcium sulfate (CaSO_4) or calcium carbonate (CaCO_3) in combination with GDL was used as a source of calcium ions to initiate gelation. When CaCO_3 was used, a CaCO_3 to GDL molar ratio of 0.5 was always maintained to achieve a neutral pH value. For all alginate solutions, a basic calcium ion to carboxyl molar ratio of 0.18 (a 36% crosslinking density for the carboxyl groups if a 100% crosslinking efficiency were achieved) was designated as 1X. The crosslinking density was adjusted with a multiplication factor to this molar ratio as a relative calcium ion content, such as 0.5X (molar ratio: 0.09), 1.5X (molar ratio: 0.27), 2X (molar ratio: 0.36), and so forth. Sodium alginate solution was added to CaCO_3 suspension, mixed and vortexed for one minute. A fresh aqueous GDL solution was then added to the

suspension and vortexed for one minute to initiate gelation. When calcium sulfate was used as a source of calcium ions, calcium sulfate dihydrate ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$) was dispersed in either deionized water or tissue culture medium. The suspension was added to sodium alginate solution and vortexed for 15 seconds. These gels were prepared in Teflon vials with dimensions of 20 mm deep and 18 mm in diameter to form circular discs, approximately 8 mm thick and 18 mm in diameter. The vials were capped, sealed with Parafilm and stored in high humidity at room temperature for 48 hours of gelation. The indicated alginate gel concentrations in this paper were the final weight/volume concentrations.

B. Homogeneity

Homogeneity of the cylindrical alginate gels was characterized with the dry to wet weight ratio against position in both axial and radial directions. Gels (8 mm thick) were cut perpendicular to the cylinder axis into 5 slices with approximately the same thickness. The slices were labeled 1 through 5 from top to bottom. After measuring their wet weights, the slices were dried at 40°C for 24 hours. The specimens were weighed again after drying and their dry/wet weight ratios were calculated. The averages and the standard deviations of triplets were reported. The homogeneity in radial direction was characterized with the dry to wet weight ratio of concentric hollow cylinders cored out of the gels (4 hollow cylinders in addition to the cylindrical core of each gel, labeled 1 through 5 from the circumference to the center). Their diameters (OD-ID) were approximately: 18.0-13.3, 13.3-12.1, 12.1-7.2, 7.2-4.1, and 4.1-0 mm respectively.

C. Mechanical Testing

Uniaxial compression was performed to measure the mechanical properties of the alginate gels with an Instron 4502 mechanical tester (Instron Corporation, Canton, MA) at a crosshead speed of 4.8 mm/min. The specimens were coated with silicone oil (Aldrich, Milwaukee, WI) to reduce the effect of friction between the gel and the plates during compression. Eight specimens (8 mm thick and 18 mm in diameter) were tested for each sample. Averages \pm standard deviations were reported.

D. Swelling Ratio

One gel disc (8 mm thick and 18 mm in diameter) was immersed in 15 ml swelling solution. For water or saline based swelling solutions, the swelling experiments were carried out at room temperature in high humidity to reduce the effects of evaporation. For tissue culture medium based swelling solutions, the swelling experiments were carried out in a humidified incubator at 37°C with 5% CO₂. The solutions were changed every other day. At the scheduled times, the specimens were picked up with a spatula and all the surfaces were quickly blotted on a Kimwipe tissue for two times. The specimens were weighed with an analytical balance accurate to 10⁻⁴ g. The whole process for each specimen was done within one minute to minimize the effects of evaporation. For each sample, triplets were tested to calculate the average values and standard deviations.

E. Cell Incorporation and Cultivation

MC3T3-E1 osteoblasts were cultured and expanded in tissue culture medium (89% DMEM containing 4500 mg/L D-glucose, 10% FBS, 1% P/S). The cultured cells were trypsinized with trypsin-EDTA and washed twice with DPBS. The cells were then suspended in sodium alginate solutions in “complete medium” (89% DMEM, 10% FBS, 1% P/S, and 50 mg/L L-ascorbic acid) at a density of 6.7x10⁶ cells/ml. The cell-containing sodium alginate solution was added to CaCO₃ suspension in Teflon vials to form circular discs 4 mm thick and 18 mm in diameter, to which a fresh aqueous GDL solution was then added to initiate the gelation. They were kept in

a humidified incubator to gel at 37°C in the presence of 5% CO₂. The vial walls were notched at the top to allow air circulation when capped. After 48 hours, the gels were transferred to 60x15 mm petri dishes and cultured in 15 ml “complete medium” supplemented with calcium chloride to obtain an desired calcium ion concentration.

5 The whole preparation was done in a biological safety cabinet (Model 1184, Forma Scientific, Inc., Marietta, OH), with aseptic techniques. Everything involved (apparatus, tools, chemicals and so on) was sterile. The medium was changed every other day.

F. Histology

10 Histologic specimens of cell-alginate constructs were embedded in paraffin, and cut into 5 mm cross sections for hematoxylin and eosin, Masson’s trichrome, or von Kossa stained analyses.

III. Results and Discussion

15 One of the requirements of compressive testing was that the surfaces of the gels be flat and parallel. In some cases, the calcium source reacted with the polymer too quickly for the surfaces of the gels to level, resulting in lumpy gels (Table 1). Mechanical properties of gels prepared with different alginates and calcium ion sources of the same polymer concentration and the same relative calcium content were compared (Table 1). *LH* alginate gels prepared with CaCO₃-GDL was the strongest of
20 the tested gels.

Table 1. Comparison of alginate gels prepared from different alginate and calcium sources of the same alginate concentration (1.5%) and calcium content (1.5X).

Alginate Source	Calcium Source	Compressive Strength (kPa)	Compressive Modulus (kPa)	Appearance
M. pyrifera	CaSO ₄ •2H ₂ O	13.3±4.50	7.49±2.33	lumpy
L. hyperborea	CaSO ₄ •2H ₂ O	36.6±17.1	5.55±2.55	soft
L. hyperborea	CaCO ₃	161±9.72	22.4±4.17	strong

A. Homogeneity

The homogeneity of cylindrical alginate gels were characterized with the dry/wet weight ratio in both axial and radial directions. *MP* alginate gels with varying polymer concentrations (1.00, 1.25, 1.50, 1.75, 2.00%) were prepared with 1X CaCO_3 . Horizontally sliced sections and vertically cored hollow cylinders exhibited comparable homogeneity profiles for all five alginate concentrations (Figure 1). This indicated that the polymer concentration did not have significant effects on the gel homogeneity in the concentration range studied. In the axial direction, the dry/wet weight ratio was almost the same everywhere except for the bottom slice. The slightly higher ratio of the bottom slice was likely due to either adherence of the CaCO_3 powder to the bottom during mixing or some sedimentation during the gelation. In the radial direction, the dry/wet weight ratio decreased slightly from circumference to the center.

B. Syneresis

Syneresis was characterized with the volume ratio of a gel to the suspension before gelation. The gels studied were *MP* alginate gels prepared with a polymer concentration of 1.75% and varying relative calcium ion contents (0.5, 1.0, 1.5 and 2.0X CaCO_3), and *LH* alginate gels prepared with a polymer concentration of 1.50% with varying relative calcium ion contents (1.0, 1.5, 2.0, 3.0, and 4.0X CaCO_3). The general trend was that the shrinkage increased with calcium concentration (Figure 2). It was assumed that an increased calcium content enhanced the inter- and intra-molecular interactions of an alginate gel, shortened the average distance between ionic crosslinks, and resulted a higher shrinkage.

C. Mechanical Properties

One of the requirements for compressive testing was that the surfaces of the gels be flat and parallel. $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ reacted with the polymer too quickly for the surfaces of the gels to level, resulting in a lumpy gels. The CaCO_3 -GDL system, on the other hand, allowed enough time for proper mixing of the suspension and leveling

of the surfaces. When $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ was used, gels were prepared slightly thicker and cut to 8 mm thick to obtain the flat and parallel surfaces for mechanical testing.

Gels made with 1.5% MP alginate and varying concentrations of CaCO_3 were compared to those made with varying concentrations of $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ (Figures 8a and 8b). The uniform gels made with CaCO_3 possessed greater modulus and strength than those made with $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$. Similar results were found with *LH* alginate gels (Figures 8c and 8d).

Mechanical properties of the alginate gels changed with structural variables. The compressive modulus and the compressive strength increased with alginate concentration in general (Figures 3 and 4). However, the compressive strength of a *MP* alginate gel of 2.0% concentration was slightly lower than that of a gel of 1.75% concentration (Figure 4a). The *MP* alginate solution with concentrations of 2.0% and higher were extremely viscous and difficult to work with. The fall in strength at this high alginate concentration was attributed to the poor mixing and the heterogeneous crosslinking network. The compressive modulus and the compressive strength increased with the relative calcium content (Figure 5 and 6) presumably due to increased crosslinking density. However, for the gels prepared from 1.5% *LH* alginate, the compressive strength of the gel with a relative calcium content of 5X was lower than that of the gel with a relative calcium content of 4X (Figure 6b). Precipitate was undetectable in the gels with 3X and lower CaCO_3 contents. At 4X and higher calcium content, precipitates were visible likely due to oversaturation. The decrease in compressive strength at 5X was attributed to the precipitation which might result in structural heterogeneity and stress concentrations, leading to lower average strength. The large standard deviation at 5X was another indication of the structural heterogeneity.

D. Swelling Ratio

One of the major concerns in using alginate gels as scaffolds for *in vitro* tissue engineering was the structural instability of the hydrogels in a tissue culture

environment. The swelling experiments were designed to understand how the ionically crosslinked alginate gels behave in various aqueous solutions, and to develop ways to control the shape and size of the gels in a tissue culture environment. Sets of three circular gel discs prepared from 3.18% *LH* alginate with 1.5X CaCO₃ were immersed in saline (0.9% NaCl aqueous solution) adjusted to varying calcium ion concentrations (Figure 7). The alginate gels swelled when the calcium ion concentration was low (0.0005 and 0.0010 M) while the gels shrank when the calcium ion concentration was high (0.0040 M). At calcium ion concentrations of 0.0020 and 0.0030 M, there was nearly no change in the gel weight over a two-week immersion experiment. Swelling experiments were also conducted with the gels (3.18% *LH* alginate with 1.5X CaCO₃) in “complete medium” adjusted to varying calcium concentrations. Again, the gels swelled at low calcium ion concentrations, but shrank at high calcium concentrations (data not shown). At a calcium concentration of 0.0030 M, the gel weight did not change significantly over immersion time. These results clearly showed that the size of ionically crosslinked alginate gels were controlled by the ion concentration of the medium.

E. Cell Incorporation and Cultivation

MC3T3-E1 osteoblasts were successfully incorporated into *LH* alginate gels with a polymer concentration of 3.18% and 1.5X CaCO₃. The complete medium was adjusted to a calcium concentration of 0.0030 M to control the gel size. Histological slides showed that the cells were uniformly distributed in the gels, assuming a spherical shape.

5 All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in the art are intended to be within the scope of the following claims.

CLAIMS

1. A method comprising:
 - a) providing:
 - i) an alginate salt,
 - ii) a source of calcium ions, and
 - iii) a calcium releasing compound;
 - b) mixing said alginate salt and said source of calcium ions to provide a mixture; and
 - c) adding said calcium releasing compound to said mixture to provide a three-dimensional crosslinked hydrogel system.
2. The method further comprising the step of:
 - d) culturing said three-dimensional crosslinked hydrogel system for growing cells *in vitro*.
3. The method of Claim 1, wherein said alginate salt is selected from the group consisting of sodium alginate and potassium alginate.
4. The method of Claim 1, wherein said alginate salt is prepared from an alginate source selected from *Macrocystis pyrifera* and *Laminaria hyperborea*.
5. The method of Claim 1, wherein said source of calcium ions is selected from the group consisting of calcium carbonate, calcium sulfate, and calcium sulfate dihydrate.
6. The method of Claim 1, wherein said calcium releasing compound is D-glucono- δ -lactone.

7. The method of Claim 1, wherein said source of calcium ions is calcium carbonate and said calcium releasing compound is D-glucono- δ -lactone, and wherein the molar ratio of said calcium carbonate to said D-glucono- δ -lactone is 0.5.

8. The method of Claim 1, further comprising the step of implanting said three-dimensional crosslinked hydrogel system.

9. The method of Claim 1, wherein said three-dimensional crosslinked hydrogel system has a thickness of between about 4 mm and about 8 mm, and a diameter of approximately 18 mm.

10. The method of Claim 1, wherein said three-dimensional crosslinked hydrogel system has a calcium ion to carboxyl molar ratio of 0.27.

11. A method for tissue engineering *in vitro* comprising:

a) providing:

i) cells,

ii) an alginate salt,

iii) a source of calcium ions, and

iv) a calcium releasing compound;

b) mixing said cells, said alginate salt and said source of calcium ions to provide a mixture;

c) adding said calcium releasing compound to said mixture to provide a crosslinked hydrogel; and

d) culturing said crosslinked gel to provide a three-dimensional crosslinked hydrogel/cell system for growing said cells *in vitro*.

12. The method of Claim 11, wherein said alginate salt is selected from the group consisting of sodium alginate and potassium alginate.

13. The method of Claim 11, wherein said alginate salt is prepared from an alginate source selected from *Macrocystis pyrifera* and *Laminaria hyperborea*.

14. The method of Claim 11, wherein said source of calcium ions is selected from the group consisting of calcium carbonate, calcium sulfate, and calcium sulfate dihydrate.

15. The method of Claim 11, wherein said calcium releasing compound is D-glucono- δ -lactone.

16. The method of Claim 11, wherein said source of calcium ions is calcium carbonate and said calcium releasing compound is D-glucono- δ -lactone, and wherein the molar ratio of said calcium carbonate to said D-glucono- δ -lactone is 0.5.

17. The method of Claim 11, further comprising the step of implanting said three-dimensional crosslinked hydrogel/cell system.

18. The method of Claim 11, wherein said three-dimensional crosslinked hydrogel/cell system has a thickness of between about 4 mm and about 8 mm, and a diameter of approximately 18 mm.

19. The method of Claim 11, wherein said three-dimensional crosslinked hydrogel/cell system has a calcium ion to carboxyl molar ratio of 0.27.

20. The method of Claim 11, wherein said cells are osteoblasts.

ABSTRACT

Methods and compositions are described that provide scaffolds for the support of cells. The scaffolds of the present invention have structural uniformity and desirable mechanical properties that make them suitable for a variety of uses, including uses for *in vitro* tissue regeneration or *in vivo* tissue or organ replacement. A method is described for controlling three-dimensional structure of the hydrogel/cell constructs under tissue culture environment.

5

FIGURE 1

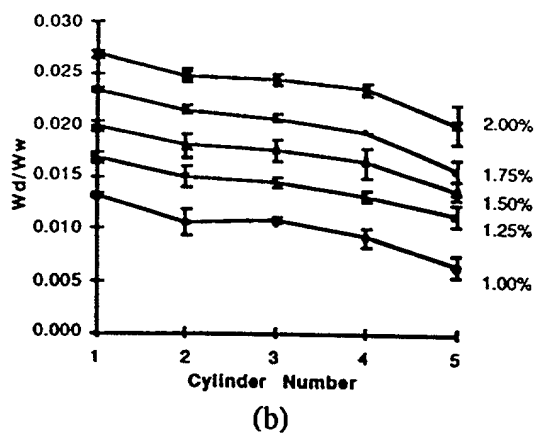
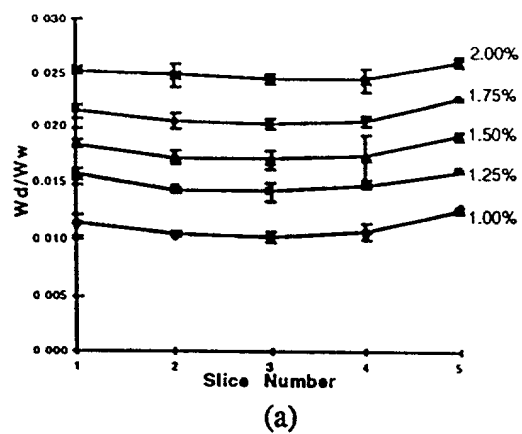


FIGURE 2

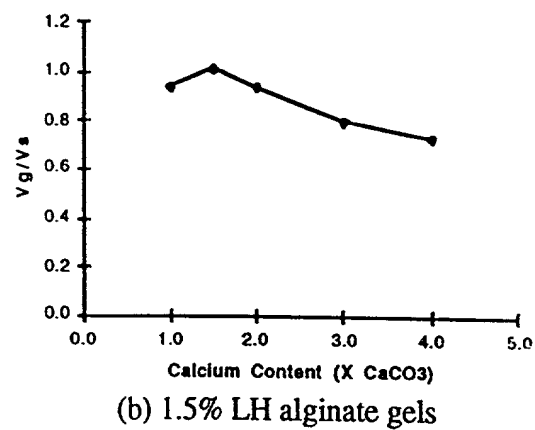
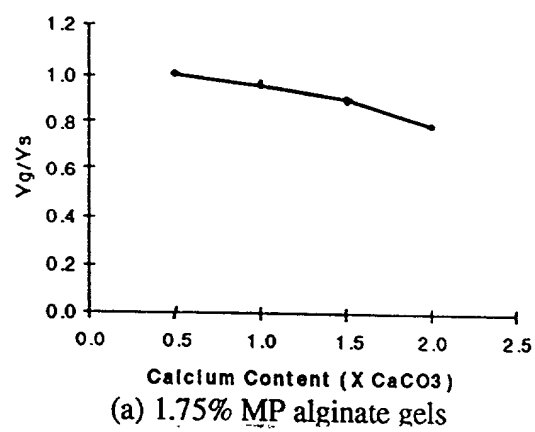
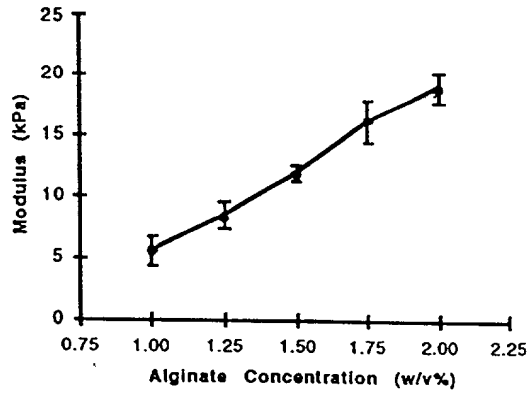
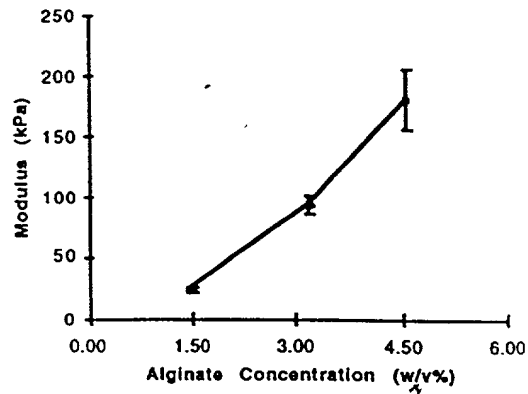


FIGURE 3

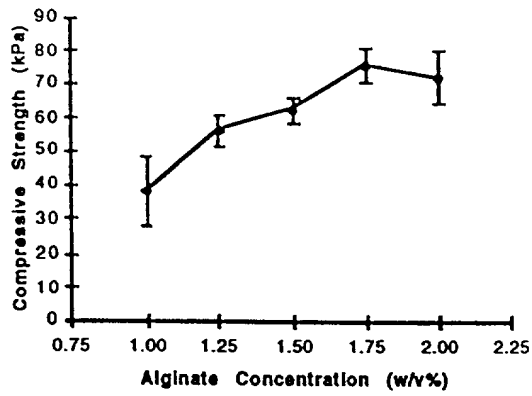


(a) MP alginate gels with 1X CaCO_3

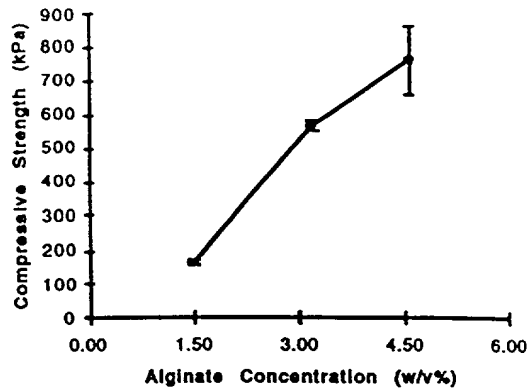


(b) LH alginate gels with 1.5X CaCO_3

FIGURE 4

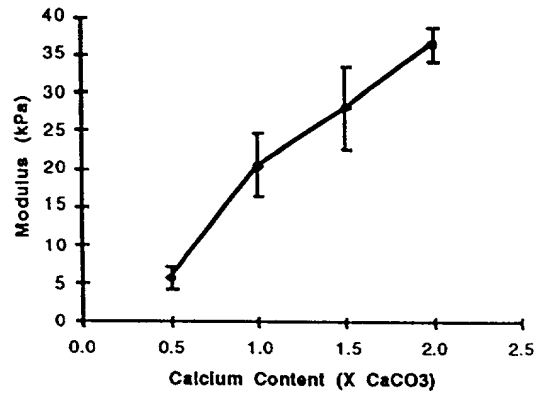


(a) MP alginate gels with 1X CaCO_3

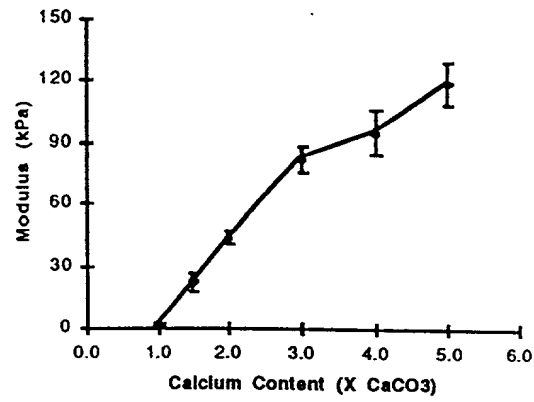


(b) LH alginate gels with 1.5X CaCO_3 .

FIGURE 5

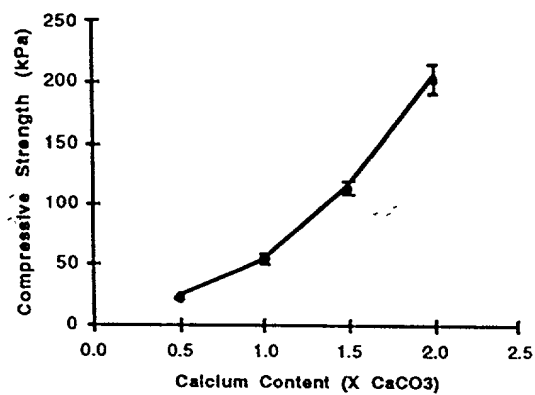


(a) 1.75% MP alginate gels.

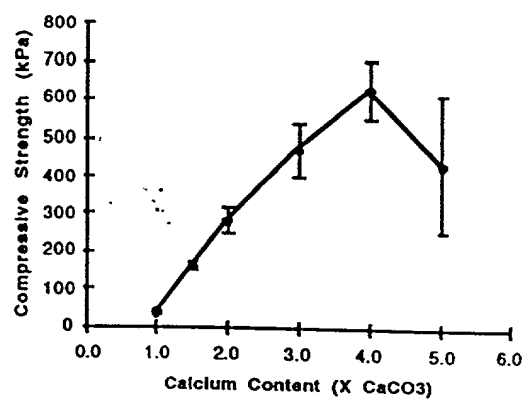


(b) 1.5% LH alginate gels.

FIGURE 6



(a) 1.75% MP alginate gels



(b) 1.5% LH alginate gels

FIGURE 7

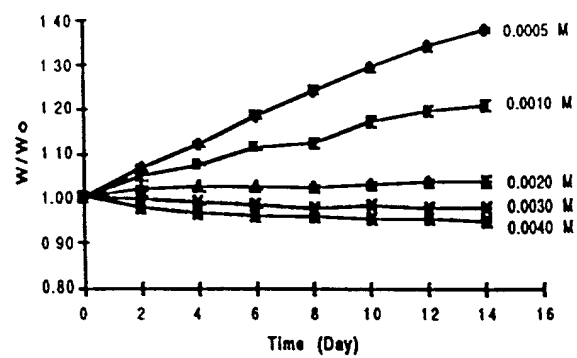


FIGURE 8a

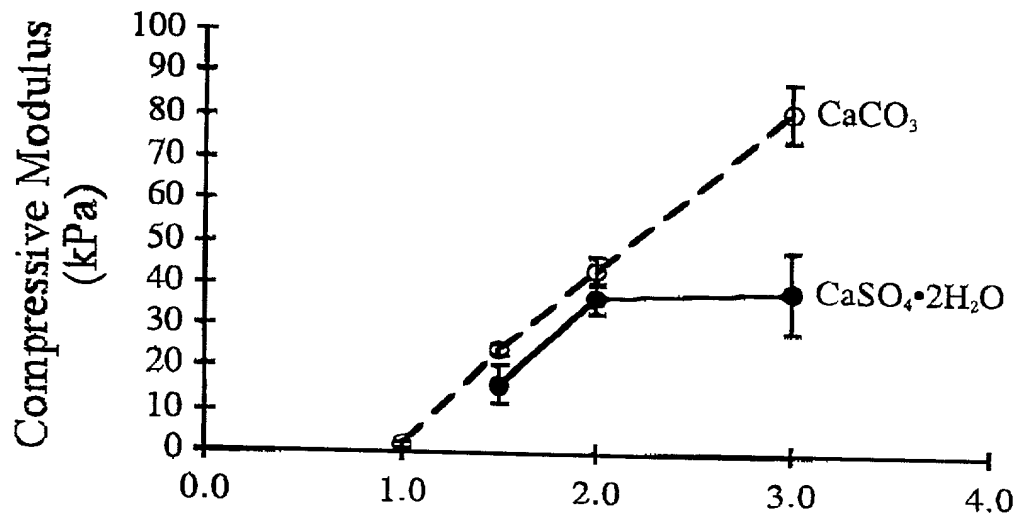


FIGURE 8b

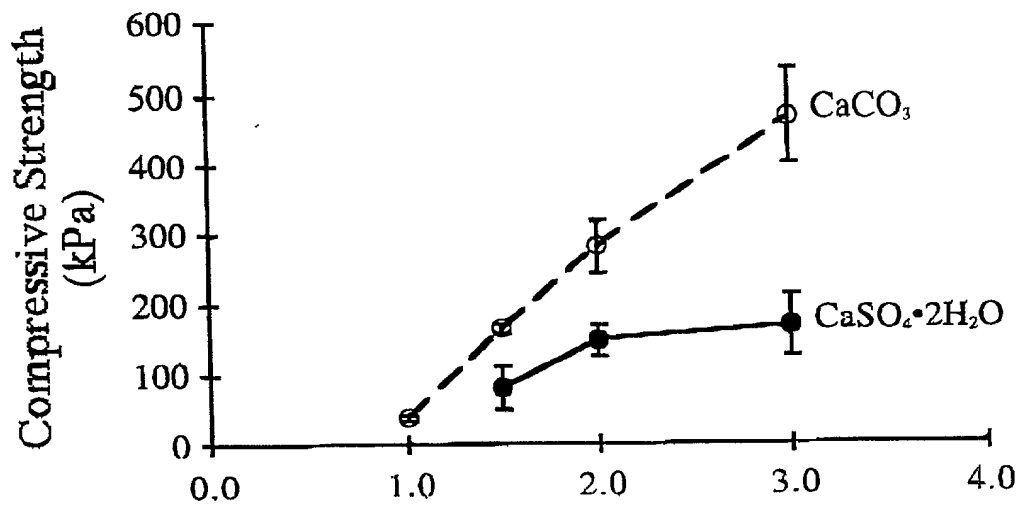


FIGURE 8c

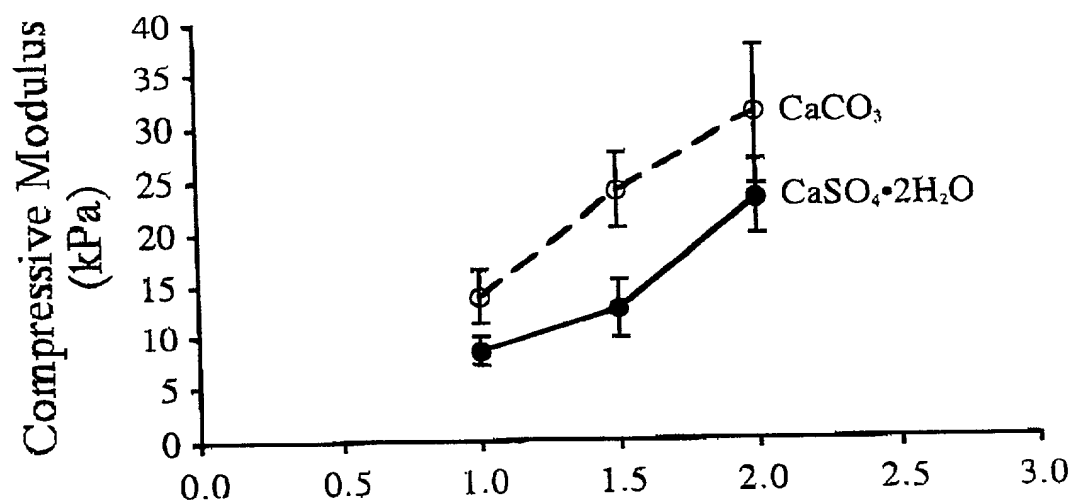
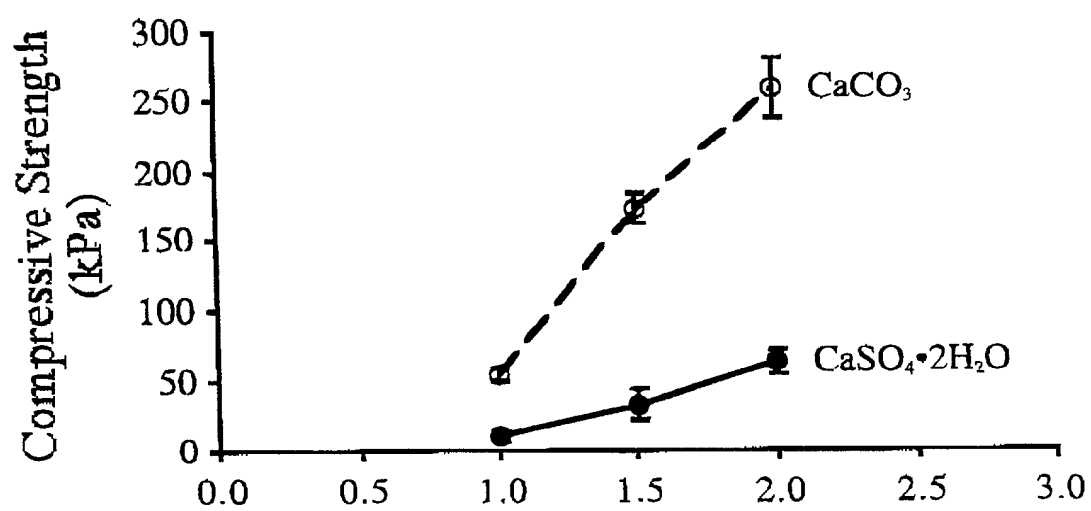


FIGURE 8d



DECLARATION FOR PATENT APPLICATION

As the below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated next to my name. I believe I am the original, first and sole inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled **THREE-DIMENSIONAL HYDROGEL/CELL SYSTEM**, the specification of which is attached hereto. I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

60/075,80202/24/98Pending*Application Serial No.**Filing Date**Patented, Pending or Abandoned*

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of Sole or First Inventor: **Peter X. Ma**

Inventor's Signature: _____

Date: _____

Residence: 3208 Foxway Court, Ann Arbor, Michigan 48105

Citizenship: China

Post Office Address: 3208 Foxway Court, Ann Arbor, Michigan 48105

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Peter X. Ma Group No.:
Serial No.: Examiner:
Filed: 02/23/99
Entitled: **THREE-DIMENSIONAL HYDROGEL/CELL SYSTEM**

POWER OF ATTORNEY BY ASSIGNEE

Assistant Commissioner for Patents
Washington, D.C. 20231

The Regents Of The University of Michigan, as Assignee of record of the entire interest of the above-identified patent application, hereby appoints the members of the firm of MEDLEN & CARROLL, LLP, a firm composed of:

Virginia S. Medlen (Reg. No. 32,050)
Peter G. Carroll (Reg. No. 32,837)
Kamrin T. MacKnight (Reg. No. 38,230)
David A. Casimir (Reg. No. 42,395)
Maha A. Hamdan (Reg. No. P-43,655)
J. Mitchell Jones (Reg. No. P-44,174)

as its attorneys with full power of substitution to prosecute this application and transact all business in the Patent and Trademark Office in connection therewith.

Please direct all future correspondence and telephone calls regarding this application to:

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220 Montgomery Street, Suite 2200 Telephone: 415/705-8410
San Francisco, California 94104 Facsimile: 415/397-8338

I hereby certify that the Assignment document filed with the application or filed subsequent to the filing date of the application, has been reviewed and I hereby certify that, to the best of my knowledge and belief, title is with The Regents Of The University of Michigan.

Dated: _____

By: _____

Name: _____

Title: _____

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